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Genome-wide Analysis of Gene Expression in Human Hepatocellular Carcinomas Using cDNA Microarray: Identification of Genes Involved in Viral Carcinogenesis and Tumor Progression[1]

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ABSTRACT

To disclose detailed genetic mechanisms in hepatocellular carcinoma (HCC) with a view toward development of novel therapeutic targets, we analyzed expression profiles of 20 primary HCCs and their corresponding noncancerous tissues by means of cDNA microarrays consisting of 23,040 genes. Up-regulation of mitosis-promoting genes was observed in the majority of the tumors examined. Some genes showed expression patterns in hepatitis B virus-positive HCCs that were different from those in hepatitis C virus-positive HCCs; most of them encoded enzymes that metabolize carcinogens and/or anticancer agents. Furthermore, we identified a number of genes associated with malignant histological type or invasive phenotype. Accumulation of such data will make it possible to define the nature of individual tumors, to provide clues for identifying new therapeutic targets, and ultimately to optimize treatment of each patient.

INTRODUCTION

Primary HCC[3] is one of the most common malignancies in the world. Despite development of novel therapeutic methods in recent years, prognosis of advanced HCC remains very poor. Major risk factors for HCC are chronic hepatitis resulting from infection with HBV or HCV, and exposure to various exogenous carcinogens including aflatoxin B1 (1). Molecular approaches have recently revealed involvement of altered *TP53*, *CTNNB1* (β-catenin), and/or *AXIN1* genes in hepatocarcinogenesis (2, 3). However, these genetic changes do not precisely reflect the biological nature of cancer cells or the clinical characteristics of individual HCC patients. Like other cancers, HCCs manifest diverse clinicopathological and biological phenotypes including grade of differentiation, proliferation rate, ability to invade vessels, potential for metastasis, sensitivity to chemotherapeutic agents, and so on. Hence, analysis of expression profiles of a large number of genes in clinical HCC materials is an essential step toward clarifying the detailed mechanisms of hepatocarcinogenesis and discovering target molecules for the development of novel therapeutic drugs.

cDNA microarray technology, which enables investigators to obtain comprehensive data with respect to gene-expression profiles, is progressing rapidly. Several studies have already demonstrated the usefulness of this technique for identifying novel cancer-related genes and for classifying human cancers at the molecular level (4, 5).

In this paper, we report the identification of genes the expression of which has been altered during hepatocarcinogenesis through the use of a genome-wide cDNA microarray containing 23,040 genes. Expression profiles of these genes in 20 primary HCCs fell into three categories that correlated well with the infection status and type of hepatitis virus. Analyses of these profiles along with clinicopathological data also facilitated identification of genes associated with tumor differentiation and vessel invasiveness. This large body of information not only furthers an understanding of the mechanisms of hepatocarcinogenesis but also reveals novel features of known genes and identifies additional biological factors involved in liver cancer.

MATERIALS AND METHODS

Patients and Tissue Samples. Primary HCCs and corresponding noncancerous liver tissues were obtained with informed consent from 20 patients who underwent hepatectomy. Patient profiles were obtained from medical records. Serologically, 10 cases were hepatitis B surface antigen-positive and 10 cases were HCV-positive. No cases with coinfections of HBV and HCV were included in this study. Histopathological classification was performed according to the Edmondson grading system; clinical stages were determined according to the Union International Contre Cancer TNM classification. No significant differences were seen between HBV-positive and HCV-positive status with respect to age, sex, grade of differentiation, vessel invasion, or tumor stage.

cDNA Microarrays. We fabricated a "genome-wide" cDNA microarray with 23,040 cDNAs selected from the UniGene database of the National Center for Biotechnology Information. The cDNAs were amplified by reverse transcription-PCR using poly(A)+ RNA isolated from various human organs as templates; lengths of the amplicons ranged from 200 to 1100 bp without repetitive or poly(A) sequences. The PCR products were spotted in duplicate on type-7 glass slides (Amersham) using an Array Spotter Generation III (Amersham). Each slide contained 52 housekeeping genes, to normalize the signal intensities of the different fluorescent dyes.

RNA Preparation, Hybridization, and Acquisition of Data. Frozen specimens were serially sectioned in 10-μm slices and stained with H&E to define the analyzed regions. To avoid cross-contamination of cancer and noncancerous cells, we prepared these two populations by laser-captured microdissection. Total RNA was extracted from each population and then amplified using Ampliscribe T7 Transcription Kit (Epicentre Technologies). The preparation of probes, hybridization, and scanning was performed as described previously (6). The fluorescence intensities of Cy5 (nontumor) and Cy3 (tumor) for each target spot were adjusted so that the mean Cy5 and Cy3 intensities of 52 housekeeping genes for each slide were equal.

Validation of Data. To assess the reproducibility of the normalized intensity ratios, we compared the $\log_2$(Cy3:Cy5 intensity ratio) of the 52 housekeeping genes between different slide sets. When the difference between normalized logarithmic ratios from two experiments was less than 1.0, we defined the data as reproducible. The reproducibility was more than 90% when the intensities of Cy3 and Cy5 were both above 25,000.

Classification of 20 HCCs According to Gene Expression Profiles. We applied the hierarchical clustering method to both genes and samples. To obtain reproducible clusters, we used only selected genes that passed the cutoff filter (both Cy3 and Cy5 signals greater than 25,000 in more than 80% cases examined). The analysis was performed using web-available software ("Clus-

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[3] The abbreviations used are: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; EST, expressed sequence tag.

Table 1 *Commonly up-regulated genes in HCC*

Among 165 genes identified as up-regulated, functions indicated for the 86 genes with official names that were up-regulated in HCCs were summarized from literature sources. Another 10 genes were categorized as having known or inferred functions according to the locus link in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/LocusLink). The remaining 69 genes were ESTs and genes with unknown function. Some genes have multiple values because multiple spots were attributed to them.

Category	Unigene	Gene name	Symbol	Locus	Function
Cell cycle					
	Hs.36708	BUB1B/Mad3L	BUB1B	15q15	controls mitotic checkpoints and chromosome segregation
	Hs.4854	cdk6 inhibitor 2c (p18)	CDKN2C	1p32	interacts strongly with cdk6, weakly with cdk4
	Hs.77597	polo (Drosophila)-like kinase	PLK	16	localizes to the mitotic spindle, involved in regulating mitotic spindle function
	Hs.77254	chromobox homolog 1	CBX1	17q	localization with centromeres in mitosis
	Hs.21635	gamma-tubulin	TUBG1	17	component of microtubule organizing center, associated with centrosome
	Hs.239	forkhead box M1/HNF-3, (MPP2)	FOXM1	12p13	control of cell proliferation, phosphorylated by M-phase kinases
	Hs.79090	exportin 1 (CRM1)	XPO1	2p16	a receptor for nuclear export signal, cell cycle regulated gene
	Hs.79101	cyclin G1	CCNG1	5q32-q34	elevation of cyclin G1 expression following DNA damage
	Hs.90073	chromosome segregation 1-like	CSE1L	20q13	chromosome segregation, importin-alpha reexport
	Hs.153346	cdc23	CDC23	5q31	a component of the APC (anaphase-promoting complex)
	Hs.77550	cdc28 protein kinase 1	CKS1	8q21	binds to the catalytic subunit of the cyclin-dependent kinases
	Hs.169840	TTK protein kinase	TTK	6q13-q21	associated with cell proliferation, functioning at mitotic spindle checkpoint
	Hs.171834	PCTAIRE protein kinase 1	PCTK1	Xp11.3-p11.23	cdc2/cdc28-related protein kinase gene family, involved in cell cycle regulation
	Hs.78466	26S proteasome subunit p31	PSMD8	19	necessary for activation of the cdc28 kinase
	Hs.279671	katanin p60 subunit A 1	KATNA1	6	Katanin is responsible for the M-phase microtubule-severing activity
MAPK pathway					
	Hs.861	MAPK3 (Erk1)	MAPK3	16p11.2	a member of a family of MAPKS that participates in cell cycle progression
	Hs.86575	MAP4K1	MAP4K1	19q13.1-q13.4	binding MAP3K1 (MEKK1) activating the JNK/SAPK kinase pathway
Transcription					
	Hs.278721	ring finger protein 5	RNF5	6p21.31	putative transcriptional factor
	Hs.182528	zinc finger protein 263	ZNF263	-	regulating transcription
	Hs.159971	BAF57/SNF5	SMARCB1	22q11.23	general transcriptional activator, component of the chromatin remodeling complex
	Hs.78869	transcriptional elongation factor (SII) A, 1	TCEA1	3p22-p21.3	fused with PLAG1 in salivary tumors (PLAG1 is fused with beta-catenin)
RNA processing					
	Hs.73984	cdc-like kinase 2	CLK2	1q31	phosphorylates SR proteins of the spliceosomal complex (control RNA splicing)
	Hs.83753	snRNP polypeptides B and B1	SNRPB	20	may have a functional role in the pre-mRNA splicing or in snRNP structure
Apoptosis					
	Hs.1578	apoptosis inhibitor 4 (survivin, EPR1)	API4	17q25	counteract a default induction of apoptosis in g2/m phase
Adhesion molecule					
	Hs.70337	immunoglobulin superfamily, member 4	IGSF4	11q23.2	homology with cell adhesion molecules NCAM1 and NCAM2
	Hs.173609	pregnancy specific beta-1-glycoprotein 1	PSG1	19q13.2	the immunoglobulin superfamily, carcinoembrionic antigen (CEA) subfamily
Cytoskeleton					
	Hs.158300	hepatoma-associated protein 1	HAP1	17q21.2-q21.3	mediate interactions among cytoskeletal, vascular, and motor proteins
	Hs.166068	villin 1	VIL1	2q35-q36	brush border cytoskeleton, abnormal distribution in intestinal glandular tumors
	Hs.76899	internexin, neuronal intermediate filament protein, alpha	INA	10	neuronal intermediate filament, involved in the morphogenesis of neurons "
	Hs.5321	ARP3	ACTR3	2	control of actin polymerization
Tumor associated					
	Hs.194351	thrombin receptor-like 2	F2RL2	5q13	thrombin and its receptor increase cancer cell invasion
	Hs.119851	glypican 3	GPC3	Xq26.1	proteoglycans, modulation of IGF2 interactions with its receptor
	Hs.119851	glypican 3	GPC3	Xq26.1	-
	Hs.119851	glypican 3	GPC3	Xq26.1	-
	Hs.81915	leukemia associated phosphoprotein p18	LAP18	1p36.1-p35	increased in leukemia and brain tumor, relate to proliferation
	Hs.210	leukocyte tyrosine kinase	LTK	15q15.1-q21.1	increased in leukemia, c-ros homologue
	Hs.85289	CD34 antigen	CD34	1q32	expressed in tumor vessels in HCC
	Hs.102482	mucin 5B	MUC5B	11p15	overexpression is associated with metastasis in non-small cell lung cancer
	Hs.119129	collagen type IV, alpha 1	COL4A1	13q34	component of basement membrane, elevation of serum type IV collagen in HCC
	Hs.80539	reticulon	RCV1	17p13.1	expressed in the lung tumor of a cancer-associated retinopathy patient
	Hs.116729	aldo-keto reductase family 1, member B11	AKR1B11	7	ARL-1 and AR are overexpressed in HCCs
	Hs.315	mucin 2, intestinal/tracheal	MUC2	11p15.5	expressed in epithelial ovarian cancer, in bladder cancer
	Hs.32989	receptor activity modifying protein 1	RAMP1	2	fused with FGFR1 in myeloproliferative disorder
	Hs.110457	Wolf-Hirschhorn syndrome candidate 1	WHSC1	4p16.3	fused to IgH in multiple myeloma with FGFR3 overexpression
	Hs.37078	v-crk sarcoma virus CT10 oncogene homolog (avian)-like	CRKL	22q11.21	may mediate the transduction of intracellular signals
	Hs.155324	matrix metalloproteinase 11 (stromelysin 3)	MPP11	22q11.23	over expressed in stromal and epithelial cells of pancreatic caronoma
	Hs.118638	annexin A3	NME1	17q21.3	expressed in lung caronoma cell lines not in normal lung
	Hs.74569	ornithine decarboxylase antizyme 2	OAZ2	15	polyamine metabolism, overexpressed in prostate cancer
	Hs.2kvan	transmembrane	STC1	8p21-p11.2	overexpression in HCC and colon cancer
	Hs.77744	RAB3A member RAS oncogene family	RAB3A	19p13.2	high expression in endocrine tumors
Miscellaneous					
	Hs.76084	lamin B2	LMNB2	19p13.3	components of the nuclear lamina, which may interact with chromatin
	Hs.278721	ring finger protein 5	HKE4	6p21.3	-
	Hs.825	3 beta-hydroxysteroid dehydrogenase	HSD3B2	1p13.1	a crucial role in the biosynthesis of all classes of hormonal steroids
	Hs.276581	cytochrome c oxidase subunit 15	COX15	10q24	involved in assembly of the backbone of c oxidase
	Hs.74217	pyrroline-5-carboxylate reductase 1	PYCR1	17q24	proline metabolic pathway
	Hs.298030	secretory carrier membrane protein	SCAMP2	15	a component of post-Golgi membranes, involved in cell surface recycling
	Hs.75262	cathepsin-O	CTSO	4q31-q32	lysosomal cysteine proteinase, papain superfamily
	Hs.78067	heat shock 27kD protein 1	HSPB1	7q21	involved in stress resistance and actin organization
	Hs.261285	pleiotropic regulator 1	PLRG1	4	-
	Hs.115370	thyroglobulin	TG	8q24.2-q24.3	involved in storage of iodine and of inactive thyroid hormones
	Hs.83974	solute carrier family 21, member 2/SLC21A2	SLC21A2	3q21	-
	Hs.150956	exostoses (multiple)-like 1	EXTL1	1p36.1	homolog to EXT1 and EXT2
	Hs.40368	adaptor-related protein complex1, sigma 2	AP1S2	-	main components of the coat surrounding the cytoplasmic face of coated vesicles
	Hs.174140	ATP citrate lyase	ACLY	17q12-q21	catalyzes the formation of acetyl-CoA
	Hs.11817	nude-type mouse 5	NUDT5	10p14-p13	cleanses the cell of potentially deletenous endogenous metabolites
	Hs.158112	protein tyrosine phosphatase D	PTPRD	9p23-p24.3	-
	Hs.75780	phosphatidylinositol glycan, class C	PIGC	1q23-q25	post-translational modification of GPI anchoring protein
	Hs.24950	regulator of G-protein signalling 5	RGS5	1q23	inhibits signal transduction by increasing GTPase activity of G protein
	Hs.151242	complement component 1 inhibitor	C1NH	11q12-q13.1	-
	Hs.150601	chymotrypsin-like protease	CTRL	16q22.1	-
	Hs.25913	peroxisomal biogenesis factor 12	PEX12	17	required for protein import into peroxisomes
	Hs.101408	branched chain aminotransferase 2	BCAT2	19q13	the catabolism of the branched chain amino acids, target of c-myc
	Hs.101408	branched chain aminotransferase 2	BCAT2	19q13	-
	Hs.26880	endothelin converting enzyme-like 1	ECEL1	2q36-q37	highly similar to metallopeptidase
	Hs.76918	Niemann-Pick disease, type C1	NPC1	18q11-q12	glycoprotein regulator of intracellular cholesterol trafficking
	Hs.158331	renin-binding protein	RENBP	Xq28	catalyzes the interconversion of n-acetylglucosamin to n-acetylmannosamine
	Hs.3281	neuronal pentraxin II	NPTX2	7q21.3-q22.1	C-reactive protein like function
	Hs.77617	nuclear antigen Sp100	SP100	2q35	anti-SP100 autoantibodies occur in patients with primary biliary cirrhosis
	Hs.171889	synaptonemal complex protein 3	SYCP3	12q	meiosis specific component of synaptonemal complex (SC)
	Hs.170290	discs, large (Drosophila) homolog 5	DLG5	10q23	MAGUK (membrane-associated guanylate kinase) family
	Hs.32981	semaphorin III/F (symbol provisional)	SEMA3F	3p21.3	deleted in lung cancer
	Hs.79162	structure specific recognition protein 1	SSRP1	11q12	binds to double-stranded DNA modified by the anticancer drug cisplatin
	Hs.170225	thymopoietin alfa	TMPO	12q22	roles in T-cell development and function
	Hs.119597	stearoyl-CoA desaturase	SCD	10	women with low activity of the enzyme have a decreased breast cancer risk
	Hs.75360	carboxypeptidase E	CPE	4q31	-
	Hs.199250	chloride channel 4	CLCN4	Xp22.3	-
Genes with function inferred					
	Hs.86122	protein "A"	-	12p13.1	
	Hs.70830	U6 snRNA associated Sm-like protein LSm7	-	19p13	
	Hs.95260	autosomal highly conserved protein	-	6p22-p23	
	Hs.87245	bcl-2 binding component 3	-	19q13	
	Hs.21050	G20 protein	-	3p21	
	Hs.50758	chromosome-associated polypeptide C	-	3q25-26	
	Hs.78089	vacuolar ATPase, 14kD	-	12	

Table 1 *Continued*

	Hs.182429	protein disulfide isomerase-related protein	2p24
	Hs.234896	geminin	6p21
	Hs.180576	KIAA 1274 protein (similar to mouse palladin)	10
ESTs and genes with unknown function			
	Hs.137476	KIAA1051	
	Hs 42949	ESTs, Weakly similar to HES1[H sapiens]	
	Hs 107125	ESTs, Weakly similar to HPBRII-7 protein [H.sapiens]	
	Hs 124402	EST	
	Hs 185708	ESTs	
	Hs 121749	ESTs	
	Hs 122614	ESTs, Weakly similar to apoptotic protease activating factor 1 [M.musculus]	
	Hs 119813	ESTs	
	Hs 8109	ESTs, Weakly similar to stm-BOP2 [M musculus]	
	Hs 132348	ESTs, Weakly similar to diaphanous 2 [H.sapiens]	
	Hs 179805	ESTs	
	Hs 134253	ESTs	
	Hs 127001	ESTs, Moderately similar to ubiquitin carrier protein E2 [H sapiens]	
	Hs 92374	Hypothetical protein FLJ20746	
	Hs 94318	ESTs, Highly similar to Mus musculus mRNA for Dutt1 protein"	
	Hs.126825	ESTs	
	Hs 167583	ESTs	
	Hs 124938	EST	
	Hs.31608	Hypothetical protein FLJ20041	
	Hs 250570	ESTs	
	Hs 102447	mRNA for TSC-22-like protein	
	Hs 123938	ESTs, Weakly similar to unknown [S cerevisiae]	
	Hs 122730	ESTs, Weakly similar to Strabismus [D melanogaster]	
	Hs 3454	ESTs, Weakly similar to KIAA0665 protein [H.sapiens]	
	Hs 31841	ESTs	
	Hs 121863	EST	
	Hs 44579	Hypothetical protein FLJ20199	
	Hs 123604	EST	
	Hs 124606	EST	
	Hs 26870	ESTs, Weakly similar to Ew-5 [M musculus]	
	Hs 8003	ESTs, Moderately similar to EST AA667999	
	Hs 119670	ESTs	
	Hs.122942	ESTs	
	Hs.124839	ESTs	
	Hs 30504	cDNA DKFZp434E082	
	-	ns22b03.s1 NCI_CGAP_GCB1 cDNA clone IMAGE:1184334	
	Hs.7104	ESTs	
	Hs.15165	DKFZP564G013 protein	
	Hs.18271	cDNA DKFZp434P1217	
	Hs.134798	ESTs, Moderately similar to TUBULIN-TYROSINE LIGASE [M musculus]	
	Hs.123599	ESTs, Moderately similar to Homo sapiens hypothetical protein FLJ10858	
	Hs 59860	ESTs, Highly similar (91%) to human HMG-17 gene	
	Hs 123177	ESTs	
	Hs.126768	ESTs	
	Hs 90828	ESTs	
	Hs 167578	cDNA FLJ11095 fis, clone PLACE1005374	
	Hs 7357	cDNA DKFZp566f1546	
	Hs.103277	ESTs	
	Hs.123218	ESTs	
	Hs.58461	ESTs	
	Hs.34790	cDNA FLJ10776 fis, clone NT2RP4000323	
	Hs.26204	Hypothetical protein FLJ20831	
	Hs.13801	Hypothetical protein FLJ10898	
	Hs.126017	EST	
	Hs.2149	human actin-like peptide mRNA	
	Hs.67619	chromosome 1 specific transcript KIAA0488	
	-	ym42c04.s1 Soares infant brain 1NIB cDNA clone IMAGE:51069	
	Hs 5076	ESTs, Moderately similar to sorting nexin 3 [H sapiens]	
	Hs.49759	ESTs	
	Hs.8518	cDNA DKFZp586L1722	
	Hs 124614	ESTs	
	Hs 127535	ESTs	
	Hs 129845	ESTs	
	Hs.214343	ESTs	
	Hs.215260	ESTs	
	Hs 32538	ESTs	
	Hs 42758	ESTs	
	Hs 124657	EST	
	-	zg75f10.s1 Soares fetal heart NbHH19W cDNA clone 399211	

ter" and "TreeView") written by M. Eisen.[4] Before applying the clustering algorithm, the fluorescence ratio for each spot was first log-transformed; then the data for each sample were centered to remove experimental biases.

Identification of Genes Responsible for Clinicopathological Factors. We first arranged the relative expression of each gene (Cy3:Cy5 intensity ratio) into one of four categories: up-regulated (ratio, >2.0), down-regulated (ratio, <0.5), unchanged (ratio, between 0.5 and 2.0), and not expressed (or slight expression but under the cutoff level for detection). We used these categories to detect changes in expression that were common among samples as well as specific to a certain subgroup. To detect differentially expressed genes, we recorded the number of samples in each category within each subgroup, for each gene. Then we calculated the *U* values of Mann-Whitney tests, which measured how the sample distributions between subgroups overlap. The number of samples within each group is counted and, according to the order of the category, the number of overlapped samples is incorporated into the *U* value. A small *U* shows that the sample distribution of the two groups is clearly separated, *e.g.*, commonly up-regulated in the HBV group and down-regulated in the HCV group. We applied a hierarchical clustering algorithm to all of the selected genes using hamming distance (edit distance).

[4] Internet address: http://www.microarrays.org/software.

RESULTS AND DISCUSSION

Identification of Genes That Were Differently Regulated in HCCs. To identify genes generally involved in hepatocarcinogenesis, we compared expression profiles between 20 HCCs and their corresponding noncancerous liver tissues by means of cDNA microarray. We excluded individual data when Cy3 and Cy5 signals were <25,000 because data were not reliable for genes giving low signal intensities (see "Materials and Methods"). When we applied a cutoff signal:intensity ratio of cancer:noncancer at 2.0 165 genes including 69 ESTs were selected as being up-regulated in 75% or more of the cases examined (Table 1). This list of up-regulated genes contained *MAP4K1* as well as *MAPK3*, suggesting that activation of the MAPK pathway is a common feature of hepatocarcinogenesis. Interestingly, expression of several genes associated with mitosis, including *CDC23, TUBG1, CBX1, CKS1, PCTK1, PSMD8, CSE1L, TTK*, and *PLK1*, was commonly increased in cancer cells. As a cell-cycle modulator, *CDC23* is a known component of the anaphase-promoting complex (APC) and leads to metaphase/anaphase transition through

Table 2 *Commonly down-regulated genes in HCC*

Among 170 genes identified as down-regulated, functions indicated for the 92 genes with official names that were down-regulated in HCCs were summarized from literature sources. Another three genes were categorized as having known or inferred functions according to the locus link in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/LocusLink). The remaining 75 genes were ESTs and genes with unknown function. Some genes have multiple values because multiple spots were attributed to them.

Category	Unigene	Gene name	Symbol	Locus	Function
Liver specific					
	Hs.53155	properdin P factor, complement	PFC	Xp11.4-p11.23	a positive regulator of the alternate pathway of complement
	Hs.53155	properdin P factor, complement	PFC	Xp11.3-p11.23	-
	Hs.189583	complement component 3	C3F	12p13	coagulating system
	Hs.1290	complement component 9	C9	5p13	coagulating system
	Hs.78614	complement component 1q binding protein	C1QBP	17p13.3	coagulating system
	Hs.1279	complement component 1r	C1R	12p13	coagulating system
	Hs.2161	complement component 5 receptor 1	C5R1	19	coagulating system
	Hs.75576	plasminogen	PLG	6q26	coagulating system
	Hs.572	orosomucoid 1	ORM1	9q34.1-q34.3	alpha-1-acid glycoprotein 1
	Hs.75792	hemoglobin, alpha 1	HBA1	16pter-p13.3	hemoglobin subunits
	Hs.75792	hemoglobin, alpha 1	HBA1	16pter-p13.3	hemoglobin subunits
	Hs.155376	hemoglobin, beta	HBB	11p15.5	hemoglobin subunits
	Hs.36977	hemoglobin, delta	HBD	11p15.5	hemoglobin subunits
	Hs.75442	albumin	ALB	4q11-q13	-
	Hs.181062	serum amyloid A1	SAA1	11p15.1	major acute phase reactant, the precursor of amyloid protein AA
	Hs.181062	serum amyloid A1	SAA1	11p15.1	-
	Hs.181062	serum amyloid A1	SAA1	11p15.1	-
	Hs.75442	albumin	ALB	4q11-q13	-
Detoxification and drug metabolism					
	Hs.2667	metallothionein 1H	MT1H	16q13	have a high content of cysteine residues that bind various heavy metals
	Hs.74170	metallothionein 1E	MT1E	16q13	
	Hs.203936	metallothionein 1F	MT1F	16q13	
	Hs.118786	metallothionein 2A	MT2A	16q13	
	Hs.118786	metallothionein 2A	MT2A	16q13	
	Hs.94350	metallothionein 1L	MT1L	16q13	
	Hs.74170	metallothionein 1E	MT1E	16q13	
	Hs.76669	nicotinamide N-methyltransferase	NNMT	11q23.1	n-methylation of nicotinamide and pyridines (biotransformation of many drugs)
	Hs.174270	cytochrome P450IIC, polypeptide 8	CYP2C8	10q24.1	mephenytoin 4-hydroxylase
	Hs.183584	cytochrome P450IIA, polypeptide 6	CYP2A6	19q13.2	phenobarbital-inducible cytochrome P450
	Hs.167529	cytochrome P450IIC, polypeptide 9	CYP2C9	10q24.1	mephenytoin 4-hydroxylase
Lipid metabolism					
	Hs.242908	lecithin-cholesterol acyltransferase	LCAT	16q22.1	central enzyme in the extracellular metabolism of plasma lipoproteins
	Hs.127510	short chain acyl-CoA dehydrogenase	ACADS	12q22-qter	catalyzes the initial step of the mitochondrial fatty acid beta-oxidation system
	Hs.76394	enoyl CoA hydratase, short chain, 1	ECHS1	10q26.2-q26.3	catalyzes the second step in the mitochondrial fatty acid beta-oxidation pathway
	Hs.76394	enoyl CoA hydratase, short chain, 1	ECHS1	10q26.2-q26.3	-
	Hs.82208	acyl-CoA dehydrogenase, very long chain	ACADVL	17p11.2-p11.1	fatty acid beta-oxidation
	Hs.1645	cytochrome P450IVA, polypeptide 11	CYP4A11	1	catalyze the omega- and (omega-1)-hydroxylation of various fatty acids
Retinol metabolism					
	Hs.77667	lymphocyte antigen 6 complex, locus E	LY6E	8q24.3	retinoic acid induced gene E
	Hs.101850	retinol-binding protein 1, cellular	RBP1	3q21-q22	intracellular transport of retinol
	Hs.101850	retinol-binding protein 1, cellular	RBP1	3q21-q22	-
	Hs.150595	cytochrome P450XXVIA, polypeptide 1	CYP26A1	10q23-q24	retinoic acid-metabolizing cytochrome P450
	Hs.158205	basic leucine zipper nuclear factor 1 (JEM-1)	BLZF1	1q23	up-regulated during retinoid-induced maturation of NB4-promyelocytic leukemia cells
Apoptosis					
	HS.156344	DNA fragmentation factor-45	DFFA	1p36.3-p36.2	inhibitor necessary for DFFB expression and stabilization in an inactive state
	HS.839	IGF binding protein, acid labile subunit	IGFALS	-	forms a ternary complex with IGF 1 or IGF 2 and IGFBP3
	HS.77326	IGF binding protein 3	IGFBP3	7p14-p12	prolongs the half-life of the IGF, induces apoptosis
	HS.77326	IGF binding protein 3	IGFBP3	7p14-p12	prolongs the half-life of the IGF, induces apoptosis
	Hs.110571	GADD45 beta	GADD45B	19p13.3	involved in the regulation of growth and apoptosis
Tumor suppressor gene					
	Hs.1845	p53	TP53	17p13.1	induces G1, G2 arrest, and apoptosis
MAPK pathway					
	Hs.180533	MAP kinase kinase 3b	MAP2K3	17q11.2	catalyzes the phosphorylation of a threonine and a tyrosine residue in the mapk p38
	Hs.171595	dual specificity phosphatase 1	DUSP1	5q34	dephosphorylates map kinase erk2, reversing the activation of MAP kinase family
	Hs.5591	MAPK interacting serine/threonine kinase 1	MKNK1	1	-
Cell cycle					
	Hs.82932	cyclin D1	CCND1	11q13	G1 cyclin
Immune system					
	Hs.181125	immunoglobulin lambda gene cluster	IGL@	-	immunoglobulin lambda gene cluster
	Hs.181125	immunoglobulin lambda gene cluster	IGL@	-	-
	Hs.107055	STAT induced STAT inhibitor 3	SSI-3	-	critical in negatively regulating fetal liver hematopoiesis
	Hs.140	immunoglobulin heavy constant gamma 3	IGHG3	14q32.33	Ig gamma-3 chain c region (heavy chain disease protein)
	Hs.140	immunoglobulin heavy constant gamma 3	IGHG3	14q32.33	-
	Hs.32225	immunoglobulin heavy constant alpha 1	IGHA1	14q32.33	the major immunoglobulin class in body secretions
	Hs.77423	stromal cell-derived factor 1	SDF1	-	the principal ligand for CXCR4, a coreceptor with CD4 for HIV-1
	Hs.179543	immunoglobulin heavy constant mu	IGHM	14q32.33	-
	Hs.24395	small inducible cytokine B, member 14	SCYB14	5q31	decreased expression in many cancer cell lines
	Hs.182611	solute carrier family 11, member 1	SLC11A1	2q35	macrophage-specific membrane transport function, controls resistance to infection
	Hs.74076	CD163 antigen	CD163	-	macrophage associated antigen
	Hs.80738	sialophorin (leukosialin, CD43)	SPN	16p11.2	major glycoproteins of T lymphocytes, plays a role in lectin binding
	Hs.84298	MHC class II antigen gamma chain	CD74	5q32	-
	Hs.52002	D5 antigen-like	CD5L	1q21-q23	expressed in lymphoid tissues, potential regulator of monocyte activation
	Hs.76325	immunoglobulin J polypeptide	IGJ	4q21	J linker for immunoglobulin alpha and mu chains
	Hs.123642	eph-like tyrosine kinase 1	EPHA3	3p11.2	receptor for members of the Ephrin-A family, could play a role in lymphoid function
	Hs.2157	Wiskott-Aldrich syndrome	WAS	Xp11.23-p11.22	possible regulator of lymphocyte and platelet function
	Hs.54443	chemokine (C-C motif) receptor 5	CCR5	3p21	expressed in lymphoid organs, a reduced risk of AIDS lymphoma with mutation
Miscellaneous					
	Hs.121555	myosin 1E	MYO1E	15q21-q22	-
	Hs.94925	dihydroorotate dehydrogenase	DHODH	16q22	catalyzes the fourth step of the pyrimidine de novo biosynthesis
	Hs.54505	aquaporin 6, kidney specific	AQP6	12q13	forms a water-specific channel
	Hs.104	HGF activator	HGFAC	4p16	serine protease involved in the endoproteolytic processing of HGF
	Hs.2017	ribosomal protein L38	RPL38	17q	-
	Hs.117367	solute carrier family 22, member 1	SLC22A1	6q26	organic cation transporter 1 (hOCT1)
	Hs.188096	E74-like factor 3	ELF3	1q32	putative regulation of epithelial cell differentiation
	Hs.1665	Zinc finger protein homologous to Zfp-36	ZFP36	19q13.1	-
	Hs.239156	syntaxin binding protein 1	STXBP1	9q34.1	implicated in vesicle trafficking and neurotransmitter release
	Hs.234234	aldolase B, fructose-bisphosphate	ALDOB	9q22.3	catalyzes the sixth step in glycolysis
	Hs.95822	zinc finger protein 262	ZNF262	1p32-p34	-
	Hs.2730	heterogeneous nuclear ribonucleoprotein L	HNRPL	-	a component of the heterogeneous nuclear ribonucleoprotein (hnrnp) complexes
	Hs.85087	latent TGF beta binding protein 4	LTBP4	19q13.1-19q13.2	associated with TGF-beta signaling
	Hs.8231	alkaline phosphatase, placental	ALPP	2q37	-
	Hs.89538	cholesteryl ester transfer protein, plasma	CETP	16q21	involved in the transfer of insoluble cholesteryl esters
	Hs.31053	cytoskeleton-associated protein 1	CKAP1	19q13.11-q13.12	-
	Hs.172744	5-hydroxytryptamine receptor 2A	HTR2	13q14-q21	involved in control of aldosterone production
	Hs.182225	RNA binding motif protein 3	RBM3	Xp11.23	-
	Hs.154145	G protein, olfactory type	GNAL	18p	-
	Hs.122647	N-myristoyltransferase 2	NMT2	10	adds myristoyl group to n-terminal glycine residue of certain cellular proteins
	Hs.75722	ribophorin II	RPN2	20q12-q13.1	essential subunit of n-oligosaccaryl transferase
	Hs.31791	acylphosphatase 2, muscle type	ACYP2	2	-
	Hs.1011	protein Z	PROZ	13	homologous with the vitamin k-dependent clotting factors with no enzymatic activity
	Hs.110708	sarcoglycan, epsilon	SGCE	7q21-q22	stabilizing the link between dystroglycan and dystrophin
Genes with function inferred					
	Hs.2719	HE4 extracellular proteinase inhibitor	-	20q12-q13.2	

Table 2 *Continued*

	Hs.155553	HNK-1 sulfotransferase	-	-
	Hs.234433	Amino acid transporter 2	-	12
ESTs and genes with unknown function				
	Hs.192989	mRNA for rearranged Ig kappa light chain variable region		
	Hs.108268	EST		
	Hs.99583	EST		
	Hs.99619	ESTs		
	Hs.62036	EST		
	-	yy62e11.s1 Soares_multiple_sclerosis_2NbHMSP Homo sapiens cDNA clone IMAGE:278156		
	Hs.100163	EST		
	Hs.116114	ESTs		
	Hs.111406	EST		
	Hs.5811	Hypothetical protein FLJ20467		
	Hs.260280	Homo sapiens clone 23623 mRNA		
	Hs.8509	ESTs, Weakly similar to C3 precursor		
	Hs.100134	ESTs		
	Hs.99552	ESTs		
	Hs.87491	ESTs		
	-	Human chromosome 17q21 mRNA clone 1046:1-1		
	Hs.117020	EST		
	Hs.117163	EST		
	Hs.111394	ESTs		
	Hs.6607	cDNA DKFZp566F164		
	Hs.32603	ESTs		
	Hs.102201	ESTs		
	Hs.113025	ESTs		
	Hs.185055	BENE protein		
	Hs.7837	cDNA FLJ10457		
	Hs.23823	EST		
	Hs.26302	ESTs		
	Hs.32234	ESTs, Weakly similar to CARS-Cyp [H.sapiens]		
	Hs.80690	EST		
	Hs.49414	ESTs		
	-	Homo sapiens genomic DNA, chromosome 21q22.1, segment 2/28		
	Hs.116775	ESTs		
	Hs.114659	ESTs		
	Hs.85956	ESTs		
	Hs.115590	ESTs		
	Hs.114288	ESTs		
	Hs.15476	Human DNA sequence from clone RP3-329A5		
	Hs.48814	ESTs		
	Hs.8268	ESTs		
	Hs.99562	ESTs		
	Hs.119977	ESTs		
	Hs.103840	ESTs		
	Hs.12896	KIAA1034 protein		
	Hs.172572	Hypothetical protein FLJ20093		
	Hs.100383	DKFZP586G1517 protein		
	Hs.113944	ESTs		
	Hs.111583	ESTs		
	Hs.118212	EST		
	Hs.116799	EST		
	Hs.250722	ESTs, Moderately similar to myeloid upregulated protein [M.musculus]		
	Hs.120882	ESTs		
	Hs.262987	ESTs		
	Hs.109616	ESTs		
	Hs.11860	ESTs		
	Hs.9225	ESTs		
	Hs.114086	EST		
	Hs.110820	EST		
	Hs.229726	EST		
	Hs.228660	EST		
	Hs.18045	ESTs		
	Hs.168640	ESTs		
	Hs.14438	ESTs, Moderately similar to histamine N-methyltransferase		
	Hs.26714	ESTs		
	Hs.27997	ESTs		
	Hs.88630	ESTs		
	Hs.99674	ESTs		
	Hs.91877	ESTs		
	Hs.98926	ESTs		
	Hs.88075	ESTs		
	Hs.93678	ESTs		
	Hs.87564	ESTs		
	cl.18814	EST		
	-	Homo sapiens cDNA clone IMAGE:880538		
	-	mRNA for putative lipoic acid synthetase, partial		
	-	HSPD03120 HM1 Homo sapiens cDNA clone NOTAVAIL03120		

degradation of cyclin B. *TUBG1* (γ-tubulin) and *CBX1* participate in centrosome formation (7, 8); *CKS1* and *PCTK1*, encoding cdc2/cdc28 kinases, are essential for activation of the anaphase-promoting complex. *PSMD8* (26S proteasome subunit p31) is reportedly responsible for activation of these kinases (9). Others have reported that *CSE1L*, *TTK*, and *PLK1* are associated with formation of the mitotic spindle (7, 10) and that *PLK1* can affect the number of centrosomes when exogenously expressed (11); overexpression of *PLK1* has been correlated with poor prognosis in a subset of human cancers (12). Our comprehensive expression data for these genes may account for a high incidence of chromosomal instability in HCC, and they suggest that promotion of the mitotic process is generally involved in hepatocarcinogenesis. Therefore, regulation of these mitosis-associated genes either by chemotherapeutic agents or by gene delivery might be an effective therapeutic strategy for HCCs.

We also looked for down-regulated genes and found 170 (including 75 ESTs) that were underexpressed in 65% or more of the HCCs examined (Table 2) when we applied a cutoff intensity ratio of cancer:noncancer at 0.5. The majority of the down-regulated genes encoded hepatocyte-specific gene products (*e.g.*, complement species, amyloid, and albumin) and detoxification enzymes (cytochrome P-450 and metallothionein families), reflecting de-differentiation of cancer cells. Regarding retinoid metabolism, *LY6E* and *RBP1*, both of which appear to play roles in retinoid-induced differentiation (13, 14) were repressed, as was *IGFBP3*, which also is involved in the retinol-mediated inhibition of HCC development (15). Because retinoid is an accepted therapy to encourage differentiation of cells in acute promyelocytic leukemia and is thought to help prevent development of HCC (16), reduced expression of these genes may play a crucial role in hepatocarcinogenesis.

We identified 69 ESTs that were frequently up-regulated and 75 that were frequently down-regulated, which indicated that a large number of genes of unknown function are also involved in hepatocarcinogenesis.

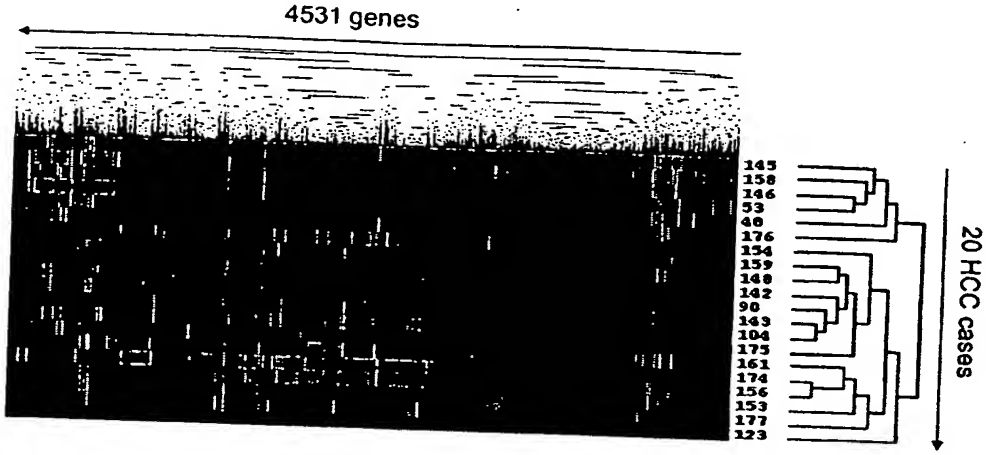

Fig. 1. Overall patterns of expression of 4531 genes across the 20 HCC samples. *Red color*, overexpression in cancer cells; *green color*, underexpression in cancer cells; *black*, unchanged expression; *gray*, no expression was detected (intensities of both Cy3 and Cy5 under the cutoff value). *Graduated color patterns* correspond to the degrees of expression changes. *Each row*, a gene; *each column*, a HCC sample. The *dendrogram of the 20 cases at the right* of the matrix indicates the degree of similarity between tumor samples demonstrating that the tumors are clustered in three groups (*red, blue*, or *green*). Sample No.123 is a very well differentiated tumor and does not appear to belong to any of the clusters. The *dendrogram at the top* also indicates the degree of similarity among the 4531 genes examined by expression patterns.

Classification of HCCs by Gene Expression Profiles. We further investigated whether clinical HCCs could be classified into groups on the basis of their gene-expression profiles. For this purpose, we used the hierarchical clustering method. To obtain reproducible clusters, we selected 4,531 genes that passed the cutoff filter (both cy3 and cy5 signals greater than 25,000). The overall expression patterns across 20 HCC samples are shown in Fig. 1. The analyses resulted in the clustering of identical genes spotted on different positions into adjacent rows, indicating the reliability of the expression data. The 20 HCCs examined fell into three groups, as the dendrogram shows.

To clarify the factors responsible for this classification, we carried out Spearman rank-correlation tests and examined clinicopathological factors including tumor differentiation, hepatitis-virus infection, TNM classification, vascular invasion, intrahepatic metastasis, and gender of the patients (data not shown). However, only the type of hepatitis virus correlated closely with these clusters ($P = 0.0079$). Therefore, HBV-positive and HCV-positive HCC may result from distinct mechanisms and be different in character as a consequence of differently expressed genes.

Identification of Genes Related to HBV-positive or HCV-positive Status. To identify genes responsible for the differences between HBV-positive and HCV-positive tumors, we performed Mann-Whitney tests and found that 19 known genes and 21 ESTs showed significantly different expression patterns between these two groups. Among the 19 known genes (Fig. 2), seven (*GPX2, CYP2E, EPHX1, AKR1C4, FMO3, UGT1A1*, and *UGT2B10*) encode key molecules for activating chemotherapeutic drugs or detoxifying xenobiotic carcinogens.

Most carcinogens are metabolized by Phase I modification enzymes that generate activated intermediates that are then detoxified by Phase II conjugation enzymes (17). Phase I enzymes *CYP2E, AKR1C4, EPHX1*, and *FMO3* convert several pro-carcinogens to activated metabolites. For example, dimethylnitrosamine is activated by *CYP2E*, and polycyclic aromatic hydrocarbons are activated by *EPHX1* and *AKR1C4* (18–20). In our study, we observed increased expression of genes encoding these four enzymes exclusively in HCV-positive HCCs, which may suggest that their enhanced expres-

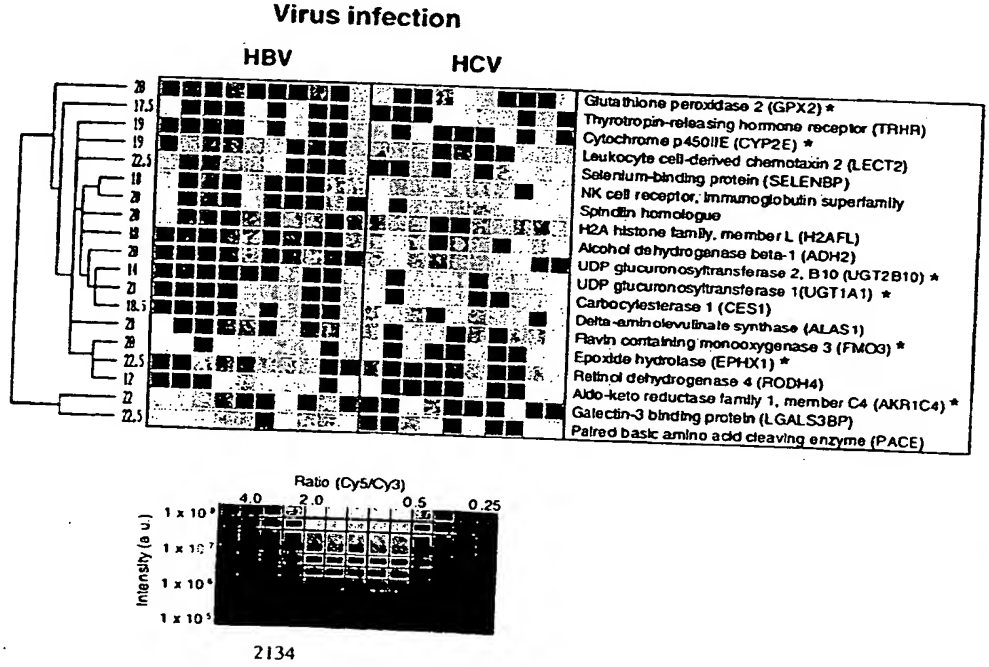

Fig. 2. Nineteen known genes of the 40 that were differentially expressed between HBV-based and HCV-based HCCs. Changes in relative expression are presented in graduated color patterns. *Red*, overexpression; *green*, underexpression; *yellow*, unchanged expression. The *number to the left of each row* is the *U* value of the Mann-Whitney test, and *the dendrogram* indicates the degree of similarity between the genes selected. *, the seven genes that encode key enzymes for detoxification of chemotherapeutic drugs or xenobiotic carcinogens.

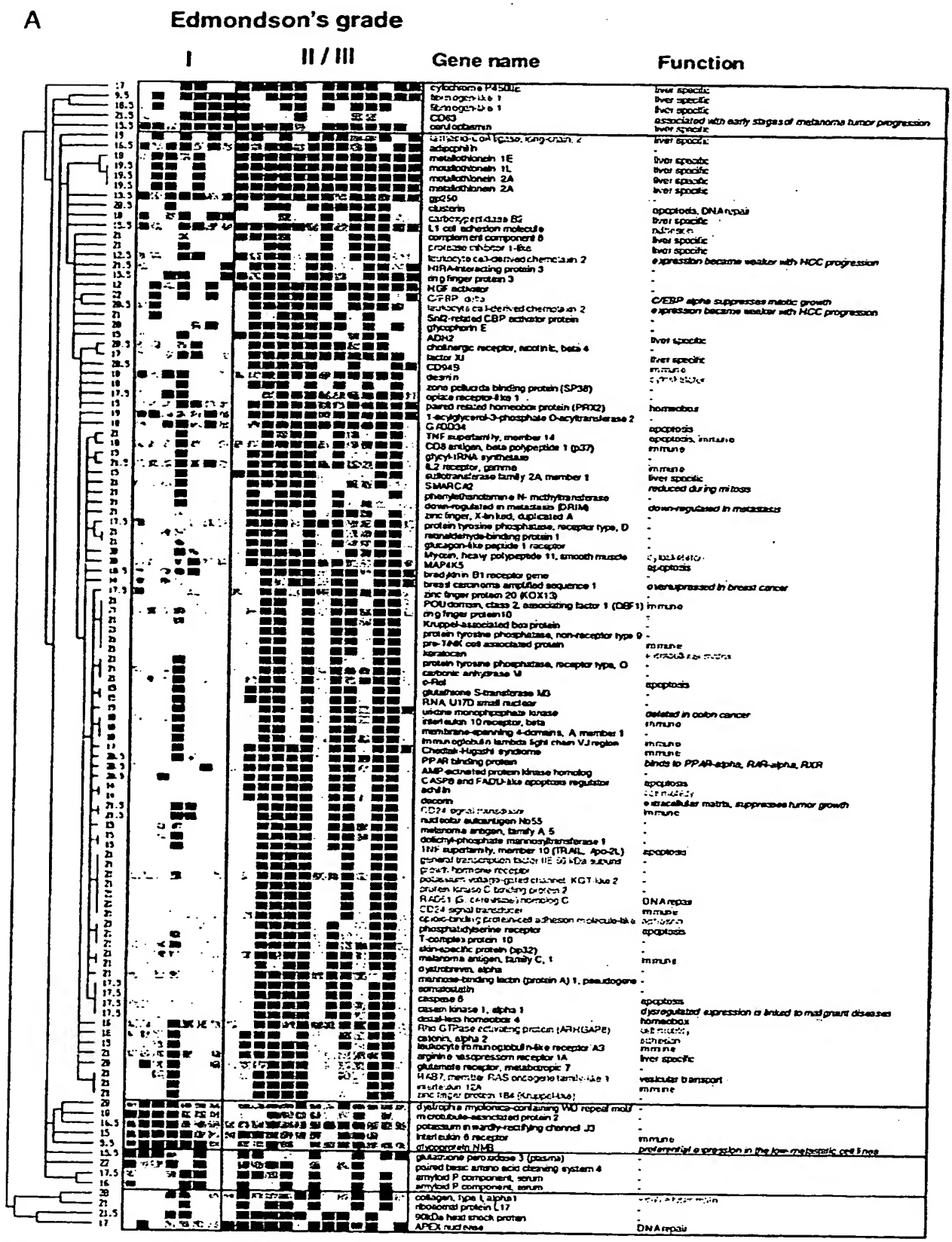

Fig. 3. Genes the expression of which is related to HCC progression. We used colors corresponding to relative gene expression as in Fig. 2. Genes related to Edmondson grade (*A*) and to vascular invasion (*B*). Among the 321 genes related to histological grade and 151 genes related to vascular invasion, 128 and 41 named genes are listed here, respectively. *Blue*, genes that are associated with both vascular invasion and grade of differentiation.

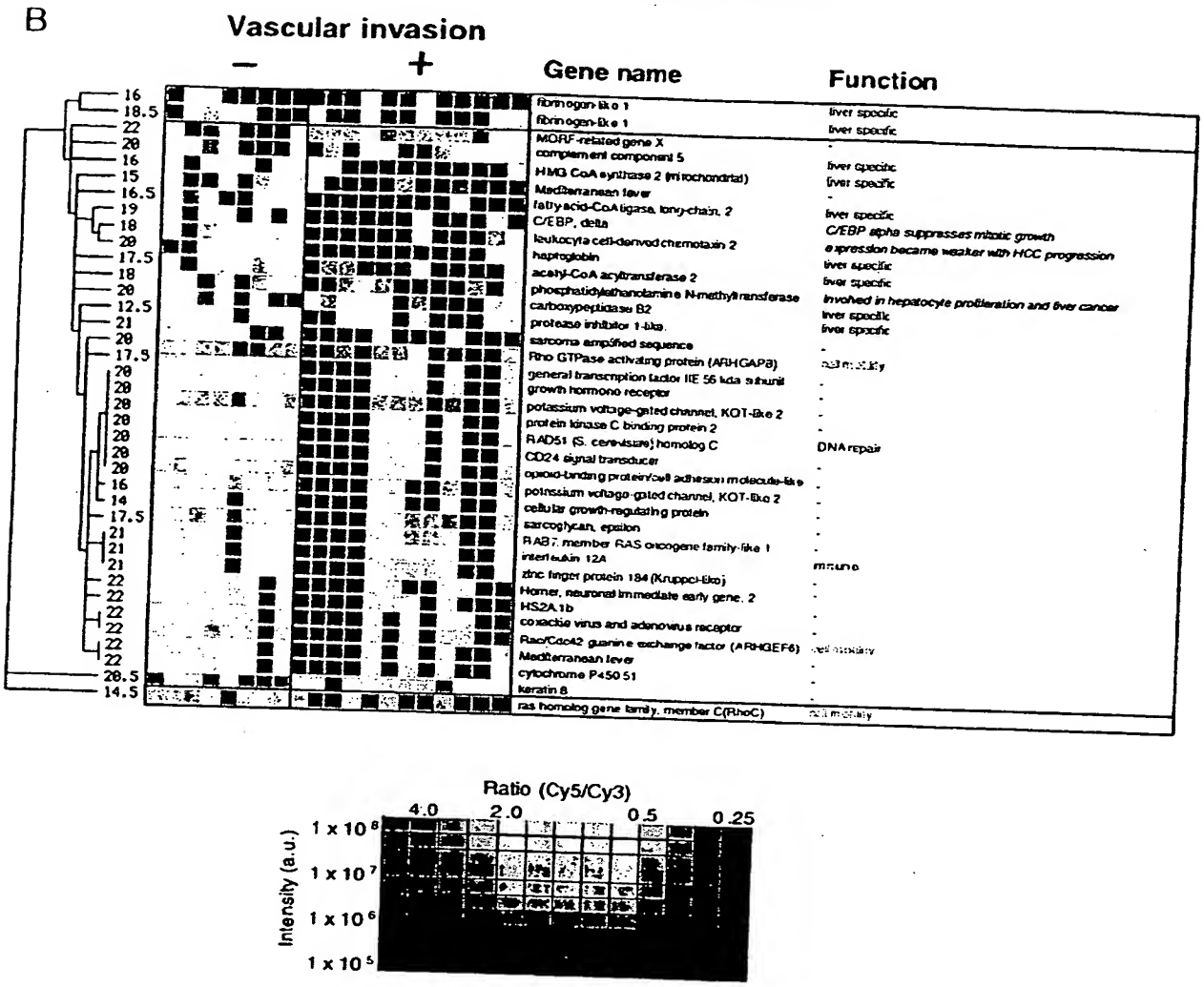

Fig 3. Continued.

sion leads to a greater contribution of carcinogenic metabolites to the mechanisms of HCV-specific hepatocarcinogenesis.

On the other hand, expression of *UGT1A1*, *UGT2B10*, and *GPX2* was preferentially repressed in HBV-positive HCCs (*UGT1A1* was reduced in 8 of 10 HBV-positive HCCs examined), but expression levels of these genes were unchanged in most HCV-positive HCCs. In accordance with our observations, Strassburg *et al.* (21) have shown decreased expression of *UGT1A1* in HCCs as well as in hepatic adenomas, implicating *UGT1A1* in an early step of hepatocarcinogenesis. *UGT1A1* and *UGT2B10* catalyze Phase II conjugation reactions, which are frequently related to detoxification of the active forms of carcinogens. GPX2, a major form of glutathione peroxidase in liver, functions as an antioxidant, and decreased glutathione peroxidase activity in HCCs has been reported elsewhere (22). Hence, reduced activities of these enzymes may reflect enhanced exposure of hepatocytes to activated carcinogens or radicals. Our results suggest that decreased expression of detoxification enzymes may be involved especially in the mechanisms of HBV-specific hepatocarcinogenesis. Furthermore, because UGT1A1 also catalyzes glucuronidation of SN-38, an active form of irinotecan (23), HBV-positive HCCs may show greater sensitivity to irinotecan than do HCV-positive HCCs. Different expression patterns among detoxification enzymes should provide information for optimizing the choice and/or the dosage of anticancer drugs for treating HCC patients on an individual basis.

Results of comparing expression profiles between HBV-positive and HCV-positive HCCs implied that hepatitis viruses affect expression of dozens of genes in HCC in a type-specific manner, invoking partly different mechanisms of carcinogenesis. Consequently, identification of genes defining virus-type-specific expression profiles would contribute to our ability to develop virus-type-dependent treatment regimens.

Identification of Genes Related to HCC Progression. As in the multistep model of adenoma-to-carcinoma sequence accepted for colorectal tumors, HCCs are considered to develop as well-differentiated tumors and then progress to moderately-to-poorly differentiated states (24). A comparison of expression profiles between well-differentiated tumors (Edmondson grade I; $n = 7$) and moderately to poorly differentiated tumors (Edmondson grade II or III; $n = 13$; Fig. 3*A*) by means of Mann-Whitney test identified a total of 321 genes (including 193 ESTs) that showed different expression patterns between the two histologically divided groups. In addition to the genes encoding liver-specific proteins, they included genes associated with apoptosis and the immune system. Apoptosis-related genes including *TNFSF10*, *TNFSF14*, *GADD34*, *CFLAR*, *CLU*, *CASP6*, and *phosphatidylserine*

receptor (25, 26) were preferentially reduced in moderately-to-poorly differentiated tumors, implying that a reduced rate of apoptosis is a major characteristic of tumor progression. Genes associated with immune systems included *MAGEC1*, one of the tumor antigens recognized by CTLs, whose expression was also repressed only in moderately-to-poorly differentiated tumors. Reduced expression of genes encoding immune targets may confer a growth advantage by allowing tumor cells to escape from immune surveillance.

Furthermore, we investigated expression profiles with respect to vascular invasiveness because vascular invasion is a major factor affecting metastasis and one of the most useful predictive factors of prognosis (27). Genes involved in vascular invasion could also represent good candidates for new therapeutic targets. We found that 151 genes (including 110 ESTs) were expressed significantly differently between noninvasive ($n = 8$) and invasive ($n = 12$) tumors (Fig. 3*B*). Among the named genes in this category, 19 were associated with both vascular invasion and tumor differentiation, but no apoptosis-related gene was among them; therefore, reduced apoptosis is likely to be correlated with tumor de-differentiation and growth, but not with vascular invasion or metastasis. Genes associated with vascular invasion contained *ARHC* (RhoC), which was recently reported to play a crucial role in metastasis (28). We also found that two other small GTPase-related genes, *ARHGAP8* (RhoGAP8) and *ARHGEF6*, were preferentially down-regulated in invasive tumors. Because the small-GTPase Rho family plays important roles in controlling cell motility and focal adhesions (29), alterations of their signaling pathways could enhance the migratory and invasive capacity of tumor cells and induce tumor invasion and metastasis. Although its function is unknown, RhoGAP8 is thought to inhibit the Rho signaling pathway; hence, reduced expression of *ARHGAP8* may also result in Rho-mediated tumor invasion. Our results suggest that controlling the Rho signaling pathway either by reducing expression of *ARHC* or by inducing *ARHGAP8* may suppress tumor invasion and subsequent metastasis.

The genes and their products represented by the numerous ESTs of unknown function that we classified in the same clusters as genes associated with apoptosis or immunity may be useful as novel targets for drug discovery or tumor markers. Accumulation of data with respect to expression profiles of cancer specimens, clinicopathological data, sensitivity to treatment, and prognosis will not only help us to understand the precise mechanisms of carcinogenesis but also yield practical information for identifying optimized therapeutic modalities and novel therapeutic targets.

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